Anti-yeast activity and characterisation of synthetic radish peptides Rs-AFP1 and Rs-AFP2 against food spoilage yeast

Laila N. Shwaiki[1], Elke K. Arendt[1,2]*, Kieran M. Lynch[1]

[1]*School of Food and Nutritional Sciences, University College Cork, Cork, Ireland*

[2]*APC Microbiome Ireland, University College Cork, Cork, Ireland*

* Corresponding author: Professor Elke K. Arendt

Mailing address: School of Food and Nutritional Sciences, University College Cork, Cork, Ireland.

E-mail address: e.arendt@ucc.ie

Short title: Synthetic radish peptides against food spoilage yeast

Declarations of interest: none

Credit Author Statement

Laila N. Shwaiki: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Writing – Review and Editing **Elke K. Arendt:** Conceptualization, Writing – Review and Editing, Supervision, Funding acquisition. **Kieran M. Lynch:** Conceptualization, Writing – Review and Editing, Supervision, Project administration.

Abstract

Food spoilage resulting from the presence of yeast is a common problem in the food industry. The development of natural food preservatives is a growing area of interest for the food industry. The application of antimicrobial peptides derived from plants can be a simple and natural method of preserving food. This study looked at the antiyeast activity of two chemically synthesised radish antimicrobial peptides, Rs-AFP1 and Rs-AFP2, for their inhibitory effect against different yeast species. The minimum inhibitory concentration (MIC) of both peptides was generated. Two mechanisms of action were studied (membrane permeabilisation and the overproduction of reactive oxygen species (ROS)) and both were found to occur with Rs-AFP2, while only the overproduction of ROS was detected for Rs-AFP1. The effect of the peptides on the yeast cells was also visualised by scanning electron microscopy. Their safety in terms of human consumption was studied and no adverse effects were found. Lastly, the stability of the peptides in different conditions, such as high salt, heat and a range of pH were studied in addition to their antiyeast activity in different food matrices such as soft drink, fruit juices and salad dressing, further supporting the peptides' potential for use in food preservation.

Keywords: Radish, defensins, antimicrobial, synthetic peptides, *Zygosaccharomyces*

1. Introduction

Antimicrobial peptides (AMPs) are a large group of host defence proteins of short amino acid sequence and positive charge (Hancock & Diamond, 2000). They are found in different life forms ranging from microorganisms to animals and humans (Adem Bahar & Ren, 2013; Jenssen, Hamill, & Hancock, 2006; Mahlapuu, Håkansson, Ringstad, & Björn, 2016). They are part of the host defence system and are antimicrobial towards a wide range of pathogens (Brown & Hancock, 2006; Hancock, 1999). AMPs isolated from plants amount to a large group of these proteins and are found in various parts of the plant, constituting part of their host defence system. Many of these peptides have been isolated from areas such as the roots, seeds, flowers, leaves and stems of plants (Goyal & Mattoo, 2016; Tang, Prodhan, Biswas, Le, & Sekaran, 2018). Defensins are one major group of AMPs in the plant kingdom. They are small proteins with 45-54 amino acids (approximately 5 kDa) and are rich in cysteine residues (De Samblanx et al., 1997a; Lay & Anderson, 2005; Neuhaus, 1999). They provide protection against fungal and bacterial pathogens during the plants' life cycle (Garvey et al., 2013).

AMPs have recently garnered increased interest in different areas of scientific research, from their integration as potential sources of novel antibiotics (Seo, Won, Kim, Mishig-Ochir, & Lee, 2012; Zaiou, 2007), to their use in food preservation (da Silva Malheiros, Daroit, & Brandelli, 2010; De Vuyst & Leroy, 2007; Schmidt, Arendt, & Thery, 2019; Shwaiki, Arendt, Lynch, & Thery, 2019). In the food industry, food spoilage can occur during stages of production (Fung, 2009), packaging (Korkeala & Johanna Björkroth, 1997) or consequentially during the storage of the food product. This contamination may be caused by the unfavourable growth of bacteria, yeast or fungal species (de W. Blackburn, 2010). Yeast species *Zygosaccharomyces*, *Saccharomyces*, *Debaryomyces* and *Kluyveromyces* are notorious for their manifestation in products such as soft drinks and salad dressing (Thomas & Davenport, 1985), wine (Kalathenos, Sutherland, & Roberts, 1995), meats and cheeses (Houtsma, de Wit,

& Rombouts, 1993; Westall & Filtenborg, 1998), and dairy products (Fleet & Mian, 1987; Mayoral et al., 2005), respectively.

The use of AMPs in the prevention of food spoilage is becoming a topic of interest in the field of bio-preservation (Ahmad et al., 2017; Cleveland, Montville, Nes, & Chikindas, 2001; Fry, 2018) as the natural aspects of using plant AMPs is appealing. Numerous plant species have been used for the extraction and purification of such AMPs (Carvalho, Machado, Da Cunha, Santos, & Gomes, 2001; Okamoto, Mitsuhara, Ohshima, Natori, & Ohashi, 1998; Tailor et al., 1997; Zhang & Lewis, 1997). The radish plant, *Ruphanus savitus*, is recognized as the source to the two defensins, Rs-AFP1 and Rs-AFP2. These two proteins are highly basic and rich in cysteine residues with a molecular weight of approximately 5 kDa (Terras et al., 1992). They have been previously extracted and purified from the seeds of the radish plant. The production of these defensins can be accomplished either from natural extraction processes using the seeds of the plant (Osborn et al., 1995; Terras et al., 1992), but can also be chemically synthesised (Koczulla & Bals, 2003). Chemically synthesising peptides can be expensive; however, a very high purity can be achieved, leading to the production of a peptide free of any unwanted compounds.

For the purpose of this study, the chemical synthesis of Rs-AFP1 and Rs-AFP2 was carried out and their activity against 5 food spoilage yeast, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis* was investigated. Their mechanism of antiyeast action was studied alongside their stability under different conditions. The safety and incorporation of the peptides into different food matrices was also explored.

2. Materials and Methods

2.1 *Rs-AFP1 and Rs-AFP2 synthesis*

Rs-AFP1 and Rs-AFP2, two homologous defensin peptides originating from the seeds of the radish plant *Raphanus sativus,* were chemically synthesised by GL Biochem (Shanghai) Ldt. Both peptides contain 51 amino acid and differ by 2 residues (Table 1). They were synthesised to a purity of 80% as indicated by the supplier. Both peptides were resuspended in water at a concentration of 2 mg/mL.

2.2 *Yeast strains*

Zygosaccharomyces bailli Sa 1403, *Zygosaccharomyces rouxii* ATCC 14679, *Kluyveromyces lactis* ATCC 56498, *Debaromyces hansenii* CBS 2334 (DMSZ (Germany)), and *Saccharomyces cerevisiae* Baker's yeast (Puratos, Belgium) were used throughout this study. Each yeast was grown aerobically in Sabouraud dextrose (SD; Sigma-Aldrich) agar at 25°C. Overnight incubation of the yeast was performed in SD broth at the same temperature under gentle agitation. All media and reagents used were obtained from Sigma-Aldrich (MO, USA), unless otherwise stated.

2.3 *Antiyeast Assays*

The minimum inhibitory concentration (MIC) of peptides Rs-AFP1 and Rs-AFP2 was determined using a microbroth dilution method as outlined by the National Committee for Clinical Laboratory Standards (NCCLS M-27A, NCCLS 2002). The yeast suspensions were prepared in SD broth from overnight cultures which were adjusted to 10^4 cfu/mL. One hundred and ninety microliters was transferred to a flat-bottom 96-well microtitre plate (Sarsdedt, Nümbrecht, Germany) followed by 10 µl of peptide ranging in concentration from 12.5 to 400 µg/mL. In order to serially dilute the peptides, 100 µL of the content in this first well was transferred in the subsequent wells containing 100 µL of yeast suspension. The positive control contained water, instead of peptide. The plates were incubated for 48 h at 25 °C in a microtitre plate reader (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA) under gentle agitation. The optical density was measured at 2 hr intervals at a wavelength of 600 nm. This assay was repeated in triplicate on 2 different plates.

In order to better visualise the peptides' antiyeast activity against *Z. bailii* over a longer period of time, an antiyeast assay was carried out and incubated for 6 days under the same conditions as above.

The fungistatic and fungicidal activity of the peptides were determined by spotting 100 µL of the yeast suspension from an antiyeast assay onto SD agar plates and incubating at 25 °C for 48-72 hr, depending on the optimal incubation time of the yeast. *D. hansenii, S. cerevisiae* and *Z. bailii* were incubated for 48 hr while *Z. rouxii* and *K. lactis* were incubated for 72 hr. The fungistatic/fungicidal activity of the peptides was determined by their ability to cause complete inhibition of the yeast.

2.4 *Colony Count Assay*

A colony count assay was performed to determine the peptides' ability to kill the yeast at different concentrations and observe the time course of this killing, as described by Jang *et al.*, 2006. This allowed for the confirmation of the peptides' activity over time. *Z. bailii* was chosen for this assay as it was the most sensitive yeast to the peptides. One millilitre of a 10^4 cfu/mL yeast suspension was prepared in SD from an overnight culture and incubated with 100 µL of peptide, ranging in concentration from 50 to 200 µg/mL. This suspension was incubated at 25

°C. One hundred microliter of the suspensions were spotted onto SD agar plates every hour over a period of 6 hr and incubated for 2 days at the same temperature. The plates were counted and a time course of the peptides' activity was determined.

2.5 *Heat, pH and Salt stability of peptides*

The peptide's stability in high heat, high salt and a range of pH was tested to determine their antiyeast activity when exposed to different environmental conditions. Peptide concentrations of 25, 50 and 100 µg/mL were tested against *Z. bailii* as the indicator yeast.

To study the effect of heat on the peptide's activity, Rs-AFP1 and Rs-AFP2 were heated for 15 min at 100°C and left to cool for 30 min before testing. An antiyeast assay was carried out as described in section 2.3.

Different ranges of pH were tested by carrying out an antiyeast assay using SD broth of which the pH was modified to 3, 5, 7, 9 and 11. The pH of the broth was modified using 1 M sodium hydroxide and 0.1 M hydrochloric acid. SD broth modified to the different pH ranges were used as a control, but without the addition of the peptides.

The stability of the peptides in different salt solutions were tested using 1 and 5 mM magnesium chloride ($MgCl_2$) and 50 and 150 mM potassium chloride (KCl). The antiyeast assay was carried out on *Z. bailii* in the presence of the four salt concentrations. Control consisted of media containing the salts without peptide.

2.6 *Membrane Permeabilisation*

The peptide's membrane permeabilisation potential was examined to determine if this was a mechanism of antiyeast action. The peptides' permeabilising activity against the cell membrane of the yeast could be detected using the dye propidium iodide, as this dye binds and stains the nucleic acids of the yeast, but this is only made possible in the case of the yeast's permeabilised membrane. A cell suspension of 10^6 cfu/mL was prepared from an overnight culture. Concentrations of peptides (10 µL) ranging from 50 to 400 µg/mL were added to 90 µL of yeast and incubated at 25°C for 2 hr. Subsequently, a final concentration of 5 µM propidium iodide (SIGMA) was added and the suspension was left to incubate at room temperature for 20 min in dark conditions, before being washed with SD broth by centrifugation at 3,000 *g* for 5 min, to remove unbound dye. Fifty microliters of these suspensions were loaded onto slides and viewed under a Confocal Laser Scanning Microsocope (CLSM) (Olympus FV1000, incorporating an IX81 inverted microscope, Germany). The negative and positive control consisted of 0.1% Triton X-100 and water, respectively. A maximal excitation (λEx) and maximum emission (λEm) wavelengths of 535nm and 617nm, respectively were used.

2.7 *Overproduction of Reactive Oxygen species (ROS)*

The overproduction of ROS by yeast in the presence of the peptides was determined using a similar assay to the membrane permeabilisation. The method established by Hayes *et al.*, 2013 was followed. A *Z. bailii* yeast suspension of 10^6 cfu/mL was incubated with 5 µg/mL of Dihydrorhodamine 123 (Sigma-Aldrich) at 25°C for 2 hr. Dihydrorhodamine 123 is an indicator dye that, in the presence of ROS, oxidises to rhodamine 123 after being taken up by the cell (Djiadeu *et al.*, 2017). After incubation, the cells were washed with SD broth by centrifugation at 3500 *g* for 5 min. Ten µL of Rs-AFP1 and Rs-AFP2 were added at different concentrations (50 to 400 µg/mL) and then incubated for 1 hr at 25°C. The cells were washed with 0.6 M potassium chloride and viewed under the CLSM by measuring the fluorescence at the maximal excitation (λEx) and maximum emission (λEm) wavelengths of 488nm and

538nm, respectively. The positive and negative control consisted of 2 mM hydrogen peroxide (H_2O_2) and water, respectively.

2.8 *Peptides' haemolytic activity*

The haemolytic activity of the peptides refers to the peptides' ability to cause the release of haemoglobin from defibrinated sheep erythrocytes, due to the cell lysis. This assay was carried out according to the method described by Thery and Arendt, 2018. Equal volumes of phosphate-buffered saline (PBS) solution was used to wash the fresh sheep's blood (Oxoid™) three times by centrifugation at 900 *g* for 15 min. This solution was made up to 4% using PBS and 80 µL was added into Eppendorf tubes in conjunction with 20 µL of peptides at different concentrations (6.25 to 400 µg/mL). The samples were incubated for 1 hr at 37°C before centrifuging them again for 10 min at 1,000 *g*. The supernatant was added into a 96 well microtiter plate and the absorption was measured at a wavelength of 405 nm. A positive control consisting of erythrocytes treated with 0.1% Triton X-100 and a negative control of PBS alone were used. The percentage of haemolysis was calculated using the absorbance measured and inputting it in the formula below.

$$\% \text{ Haemolysis} = \frac{(A405\ peptide\ treatment)-(A405\ PBS)}{(A405\ 0.1\%\ Triton\ X-100)-(A405\ PBS)}$$

2.9 *Peptides' cytotoxic activity*

The peptides' cytotoxic activity against Caco-2 cells, a colonic cell line, was performed as described by Thery *et al.*, 2019. Caco-2 cells (ECACC) were passaged in Dulbecco's Modified Eagle Media (DMEM) supplemented with 1% non-essential amino acids and 10% fetal bovine serum (FBS) and diluted to $1x10^5$ cells/mL. Two hundred microliters of this cell solution was added into wells of a flat-bottom 96 well microtitre plate and incubated for 24 hr at 37 °C with

5% CO_2. This media was removed and the two peptides were added at different concentrations in conjunction with DMEM with 2.5% FBS, bringing the volume in each well to 200 µL. The peptides were tested at concentrations of 100 to 600 µg/mL. A control consisting of sterile water and DMEM with no peptide was also tested. The plate was incubated for 24 hr at 37 °C. Subsequently, the media was removed and 100 µL of DMEM and 10 µL of MTT labelling reagent (Cell proliferation Kit I MTT; Sigma, Ireland) were added to each well and incubated for 4 hr. This was followed by the addition of 100 µL solubilisation buffer and overnight incubation. The viability of the cells were measured using a fluorometric spectrophotometer at 570 nm with a background reading of 690 nm. The assay was carried out on triplicate samples for each peptide at each concentrations.

2.10 *Peptides' resistance to proteolytic digestion*

The peptides' resistance to proteolytic digestion was tested with α-chymotrypsin (Sigma, St Louis, MO, USA), a common digestive enzyme found in the human gut. The assay was carried out as described by Thery *et al.*, 2019, in order to try and mimic the environment that the peptides may encounter if used as preservatives in food and subsequently digested. The peptides were incubated at concentrations in conjunction with α-chymotrypsin at different peptide: enzyme molar ratios of 60:1, 250:1, 2500:1, for 4 hr at 37 °C. The α-chymotrypsin was then inactivated by heat at 80 °C for 10 min before an antiyeast assay testing the peptide at concentrations of 50, 100, 200 and 400 µg/mL was performed against *Z. bailii*. The α-chymotrypsin was stored in solution in a digestion buffer consisting of 50 mM Tris–HCl (pH 7.4) and 5 mM $CaCl_2$.

2.11 *Scanning Electron Microscopy*

In order to visualise the peptides' effect on the growth of *Z. bailii* cells, samples were prepared by following the protocol of Murtey and Ramasamy, 2016. A yeast cell suspension of $1x10^6$ cfu/mL was prepared from overnight cultures. The peptides were added to individual 1.5 mL micro-centrifuge tubes containing a total of 1 mL yeast suspension (to peptide concentrations of 400 µg/mL) and incubated for 0 and 4 hr before centrifuging at 900 *g* for 2 min for fixation. The pellet was resuspended in 5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2). After 30 min, the glutaraldehyde solution was removed by centrifuged and the pellet was washed twice with 0.1 M phosphate buffer (pH 7.2). A series of ethanol washes comprising of 35%, 50%, 75%, 95% and absolute ethanol were carried out in order to dehydrate the samples. For each step, the samples were left for 30 min before centrifuging and resuspending the pellet. The last two ethanol washes comprising of 95% and absolute ethanol were repeated twice, before the addition of the first round of hexamethyldisilazane (HDMS) for another 30 min. The supernatant of the second round of HDMS was discarded and the samples were left in a desiccator to dry overnight. These were then fixed onto plain aluminum stubs and coated with a 5 nm gold-palladium layer (80:20) using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England), and viewed under a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan), under constant accelerating voltage of 5 kV. Samples containing yeast with no peptide were used as a controls.

2.12 *Peptides' application in different food matrices*

The application of Rs-AFP1 and Rs-AFP2 were assessed in different food matrices. *Z. bailii* is a yeast commonly known to spoil foods of high sugar and salt content such as salad dressings, soft drinks, syrups and wines (Blackburn, 2006; Kuanyshev, Adamo, Porro, & Branduardi, 2017). The antiyeast effect of Rs-AFP1 and Rs-AFP2 were investigated in some of these different foods.

The soft drink tested was Fanta Orange (Coca-Cola, Ireland). This was done via the microtiter plate method using filter sterilised Fanta orange inoculated with 10^2 cfu/mL yeast from an overnight culture of SD broth. This concentration of yeast was used in order to represent the number of cells found to spoil such beverages. The peptide concentrations tested ranged from 50 to 400 µg/mL. The antiyeast activity of the peptides in the Fanta Orange was measured by observing growth of the yeast over 48 hr and measuring the optical density at 620 nm. Controls consisted of Fanta Orange inoculated with 10^2 cfu/mL of yeast without peptide and Fanta Orange with no yeast added. The pH of the Fanta Orange was recorded as 3.1, a pH lower than what was found for the SD broth (pH 5.3). This protocol was also used to test the antiyeast activity of the peptides in orange juice (*SuperValu* Chilled Orange Juice) (pH 3.86), apple juice (*CYPRINA*, Apple Juice) (pH 3.54), and cranberry juice (*SuperValu*, Chilled Cranberry Juice) (pH 2.7). The controls of each beverage consisted of the corresponding beverage inoculated with 10^2 cfu/mL of yeast and no peptide.

Salad dressing (*MILANO* House Light Dressing) (pH 3.1) was used to test the peptides' activity in a more viscous food matrix. A sample of the salad dressing was inoculated with 10^2 cfu/mL of *Z. bailii* in conjunction with 400 µg/mL of each peptide. This solution was thoroughly mixed before spreading 100 µL of each sample onto SD agar plates and incubating for 3 days at 25 °C. This allowed for the determination of the peptides' effectiveness in such a viscous matrix. In order to observe the long term effect of the peptides in this food, the assay was repeated with the exception of incubating the peptides in the salad dressing for 48 hr before spreading the solutions onto SD agar plates. For both assays, salad dressing with no peptide was used as the control. The cranberry juice was subjected to the same treatment and spread onto SD agar plates in order to better visualise the peptides' antiyeast effect over 48 hr and to further confirm the results of the micro broth dilution assays in this beverage.

3. Results

3.1 *Minimum inhibitory concentrations*

The results of the antiyeast assay revealed Rs-AFP1 to be less potent than Rs-AFP2 (Table 2). *Z. bailii* was the most sensitive yeast with MICs ranging between 25 and 50 µg/mL for both peptides. *Z. rouxii* and *D. hansenii* were only inhibited by Rs-AFP2 at concentrations ranging between 50 and 100 µg/mL, and *S. cerevisiae* and *K. lactis* were not affected by either peptide, even at the highest concentration of 400 µg/mL.

The fungistatic/fungicidal activity of the peptides was determined. The only fungicidal activity observed was with Rs-AFP2 against *Z. bailii* at the highest concentration of 400 µg/mL. Rs-AFP1 was found to only be fungistatic against *Z. bailii,* the only yeast sensitive to the peptide. Rs-AFP2's fungistatic activities was also observed against both *Z. rouxii* and *D. hansenii.*

Performing an antiyeast assay for 6 days resulted in the same inhibitory effect of the peptides against *Z. bailii*. At the concentration range of the peptides' MIC (25 to 50 µg/mL), they were able to cause inhibition and continue to do so over the 6-day incubation period.

3.2 *Colony Count Assay*

The rate of *Z. bailii* inhibition affected by the peptides over 6 hr was observed (Figure 1A and 1B). Rs-AFP2 fully inhibited *Z. bailii* at all three concentrations (50, 100, and 200 µg/L) after 2 hr of incubation. The same effect can be seen at 100 and 200 µg/mL of Rs-AFP1, with the exception of 50 µg/mL only causing a reduction in *Z. bailii* after 4 to 5 hr. Increase growth of the yeast was seen for the first 3 hr, before the decrease was observed.

3.3 *Peptides' stability*

After being subjected to thermal treatment of 100 °C for 15 min, no change in their antiyeast activity was observed. At the concentration of 50 and 100 µg/mL, the peptides were able to inhibit *Z. bailii*, while at 25 g/mL there was no inhibition observed, as predicted from the antiyeast MIC assays.

The modification of the media's pH resulted in changes to the peptide's antiyeast activity to various degrees (Table 3). At pH 3, a decrease in their activity was observed from the complete growth at all concentrations tested (25, 50 and 100 µg/mL). At pH 5, both Rs-AFP1 and Rs-AFP2 were unaffected. At pH 7, a complete loss in the peptides' activity was observed. pH 9 and 11 showed no yeast growth in the controls of unadjusted SD broth, indicating that the yeast was unable to grow in such basic conditions.

Rs-AFP2's activity in the salt-containing media showed a decrease in antiyeast activity at the higher concentration of $MgCl_2$ (5mM) and in both concentrations of KCl (50 and 150 mM) (Figure 2A). At the highest peptide concentration of 100 µg/mL in 1 mM $MgCl_2$, there was considerably less yeast growth compared to 100 µg/mL in 5 mM $MgCl_2$ and 50 and 150 mM KCl. Rs-AFP1 was effected more; even 1 mM $MgCl_2$ affecting its antiyeast activity (Figure 2A). At 100 µg/mL, *Z. bailii* growth was not completely repressed, as the OD is nearly half the salt control but not low enough to consider inhibition to be occurring.

3.4 *Peptides' mechanism of action*

The permeabilisation of the yeast membranes and the overproduction of Reactive Oxygen Species (ROS) were found to be the peptides' primary mode of action against *Z. bailii.*

At the highest concentration tested (400 µg/mL), Rs-AFP2 was found to completely permeabilise the yeast (S1A). The level of permeabilisation decreased as the concentration of peptide was lowered (200 and 100 µg/mL) (S1B and S1C). At the minimum concentration tested (50 µg/mL) there was no visible permeabilisation.

Rs-AFP1 did not result in the permeabilisation of the yeast membrane, even at the highest concentration of 400 µg/mL (Result not shown).

The overproduction of ROS in the yeast due the action of the peptides was observed for both Rs-AFP1 and Rs-AFP2. As predicted, the higher concentrations of both peptides (400 µg/mL) produced a higher level of ROS formation in the yeast cells, when viewed by CLSM, in comparison to the yeast subjected to 100 and 200 µg/mL of both peptides (S2A, S2B and S2C). At 50 µg/mL, no ROS overproduction was detected (image not shown).

3.5 *Scanning Electron Microscopy*

The effect of both peptides on the yeast cells morphology showed that the peptide caused the cells to shrink. The control without the presence of the peptides produced visibly healthy and large yeast cells (Figure 5A), in comparison to the yeast inoculated with the peptides, where considerably smaller cells were observed, even at 0 hr (Figure 3B and 3D) and more considerably after 4 hr of incubation (Figure 3C and 3E).

3.6 *Peptides' haemolytic effect*

Both peptides were tested for their haemolytic activity against erythrocytes from fresh sheep's blood. At concentrations of 200 and 400 µg/mL, the peptides were found to be significantly haemolytic, with a percentage of haemolysis of more than 50% (Figure 4); 100 µg/mL and lower concentrations resulted in very low haemolysis of erythrocytes, with <10% haemolysis observed.

3.7 *Peptides' cytotoxic effect against Caco-2 cells*

The cytotoxicity assay indicated that the peptides caused an increase in the cell viability (Figure 5A and 5B), which was proportional to the concentration of the peptide added.

3.8 *Peptides' resistance to proteolytic digestion*

The peptides' resistance to proteolytic digestion by α-chymotrypsin was tested at different molar ratios of peptide: enzyme 60:1, 250:1 and 2500:1. At all molar concentrations, the protease degraded Rs-AFP1 as yeast growth was apparent at all four peptide concentrations (50, 100, 200 and 400 µg/mL). Rs-AFP2 was found to be resistant to the lowest molar ratio of 1:2500 at 400 µg/mL of peptide, as *Z. bailii* inhibition was observed, while the lower concentrations (50, 100 and 200 µg/mL) did not have any effect on the yeast, indicating the degradation of the peptide. At the higher molar ratios of 60:1 and 250:1, the peptide's antiyeast activity was completely eliminated.

3.9 *Peptides' applications in food*

Rs-AFP1 and Rs-AFP2 were tested for their antiyeast activity in different food matrices (Table 4). For the beverages, the peptides were effective in causing full *Z. bailii* inhibition in both the cranberry juice and Fanta Orange. In the apple juice, 200 and 400 µg/mL of peptide caused inhibition, while full yeast growth was observed at 50 and 100 µg/mL. The peptides were ineffective in the orange juice, as they were incapable of preventing the growth of *Z. bailii* at any of the concentrations tested. The peptide application in the salad dressing caused a reduction if yeast cells after immediate inoculation of the dressing/yeast mixture with the peptides (Figure 6). Incubating the peptides into the salad dressing with the yeast for 48 hr resulted in the yeast's full inhibition of the whole time period. Similar results were obtained for the cranberry juice after incubating for 48 hr; no yeast growth was observed in the samples containing the peptides (at 50, 100, 200 or 400 µg/mL).

4. Discussion

The synthesis of plant antimicrobial peptides through chemical means can be a more direct alternative to the extraction and purification process required to isolate these peptides from plant matrices - a process that can be laborious and time consuming. The chemical synthesis of two radish defensins, Rs-AFP1 and Rs-AFP2, was performed for this study by replicating the peptides' naturally encoded amino acid sequence (Table 1). This paper examines the antiyeast activity of these two defensin peptides, for the inhibition of some common food spoilage yeast and their application in different food matrices.

The peptides were tested for their antiyeast activity against 5 different yeast with inhibition observed against *Z. bailii*, *Z. rouxii* and *D. hansenii*. The ranges of MICs were observed to be between 25 and 50 µg/mL for both peptides against *Z. bailii* and from 50 to 100 µg/mL for Rs-ASP2 against *Z. rouxii* and *D. hansenii*. Rs-AFP1 did not show any inhibitory effect against *Z. rouxii*, *S. cerevisiae*, *K. lactis* or *D. hansenii.* The differences in this inhibitory effect against the yeast may be explained by the 2 amino acid residue difference between the peptides. The amino acid residues Asparagine-5 and Glutamate-26 found in Rs-AFP1 are replaced by Glutamine-5 and Argenine-26, respectively, in the sequence of Rs-AFP2. These slight changes result in Rs-AFP1 being less basic and having a lower net positive charge compared to Rs-AFP2. Rs-AFP1 is known to have a net charge of + 4 while that of Rs-AFP2 is + 6. This higher net charge and Rs-AFP2's additional positively charged residues could be the reason for its increased antiyeast activity. It is well known that antimicrobial peptides with a higher net charge and increased number of positively charged amino acid residues result in increased antimicrobial activity (Dathe & Wieprecht, 1999; Hong, Park, & Lee, 2001; Jiang et al., 2008; SD, 2014). The colony count assay further confirmed this difference in antiyeast activity, where, at the same peptide concentration, Rs-AFP1's inhibitory effect was apparent after a longer incubation period compared to Rs-AFP2.

The peptide sequence alignment of Rs-AFP1 and Rs-AFP2 reveal a pattern of cysteine residues that are involved in the production of 4 disulphide bridges (Fant, Vranken, Broekaert, & Borremans, 1998) and a cysteine stabilised α-β motif (De Samblanx et al., 1997b; Maróti, Downie, & Kondorosi, 2015; Van Der Weerden, Bleackley, & Anderson, 2013). The presence of multiple cysteine residues could explain the peptides' activity against the yeast. It has been reported that peptides rich in cysteine and/or glycine residues have significant antimicrobial properties (Goyal & Mattoo, 2016; Haag et al., 2012; Maróti et al., 2015). AMPs like Rs-AFP1 and Rs-AFP2 are known to effect cell membrane surfaces through the attraction of negatively charged molecules by their cationic residues (Pelegrini & Franco, 2005; Titarenko, López-Solanilla, García-Olmedo, & Rodríguez-Palenzuela, 1997; Whitlow & Teeter, 1985). This attraction ultimately causes the peptides' accumulation on the surface of the cell membrane, resulting in the potential modification of the surface, eventually leading to its death (Goyal & Mattoo, 2016). The presence of lysine (4 in each peptide) and arginine residues (2 in Rs-AFP1 and 3 in Rs-AFP2) in these peptides are the contributing residues for this interaction between the peptides and cell membrane (Sato & Feix, 2008; Yeaman & Yount, 2003), with glutamate (2 in Rs-AFP1 and 1 in Rs-AFP2) also contributing to this attribute (Goyal & Mattoo, 2016). (Illustrative scheme can be seen in S3).

Due to the intended use of the peptides in food preservation, it is important that they are able to withstand different treatments and conditions to which they may be subjected. At high temperatures, a common process encountered in food preparation, both Rs-AFP1 and Rs-AFP2 were unaffected, causing inhibition even after being subjected to 100 °C. Adjusting the pH of SD media to pH 7 resulted in the greatest change to be the peptides' activity as a loss of antiyeast action was observed. This reduction in antiyeast activity could be due to the change in the peptides' net charge that occurs at neutral and basic pH. The net charge of many cationic AMPs have been studied and observed to be more positively charged at/below pH 7

(Walkenhorst, Klein, Vo, & Wimley, 2013). The salt concentrations tested in the stability assay were based on previous papers that have looked into the stability of peptides in high salt conditions (Betts, Linton, & Betteridge, 1999; Wu et al., 2008). Rs-AFP1 in the presence of salts $MgCl_2$ and KCl resulted in a reduced ability to inhibit *Z. bailii.* This was also observed for Rs-AFP2 at the highest concentrations of the salts. This could be explained by the cations present in the medium interacting with the yeast cell membrane and potentially altering the peptides' overall charge, thus modifying their structure (Baldauf et al., 2013) and leading to the peptides' reduced antiyeast activity. Relative to Rs-AFP1, Rs-AFP2's unchanged antiyeast activity at the lowest concentration may be due to its more basic nature being a potential factor for its resistance to the salt conditions (Terras et al., 1992).

Both peptides were observed to cause the overproduction of ROS in *Z. bailii,* with the level of overproduction being dose dependant. ROS is known to be generated by yeast cells during normal cell functions, however, under cell stress conditions, the level of ROS increases dramatically, leading to an overproduction in ROS and, ultimately, cell death (Wang et al., 2015). This induction of endogenous ROS has been previously reported to occur in yeast cells and is a recognised mechanism of action of cationic AMPs (Aerts et al., 2007). The more common mechanism of action of AMPs, however, is through the permeabilisation of cell membranes. Rs-AFP2 was shown to permeabilise the cell membrane of *Z. bailii,* while Rs-AFP1 could not. This peptide's increased cationic and amphipathic nature, compared to Rs-AFP1, could explain the level of permeabilisation observed. These attributes enable the peptide to interact with the negatively charged yeast membrane and cause permeabilisation (Kumar, Kizhakkedathu, & Straus, 2018).

Analysing the peptides' inhibitory effect against *Z. bailii* under an electron scanning microscope allowed for the visual observation of their effect on the yeast cells. Cells that were treated with both peptides were observed to have a shrunken nature compared to the untreated yeast. This reduction in the size of the treated cells could potentially be due to the leakage of potassium ions, an important component required for the growth and survival of the yeast cell (Enríquez-Freire, López, & Peña, 1999; Lee & Lee, 2015; Peña, Sánchez, & Calahorra, 2013). Studies have shown that cationic plant antimicrobial peptides, like Rs-AFP1 and Rs-AFP2, can cause this rapid efflux of potassium ions as a result of membrane damage (De Samblanx et al., 1997b; Enríquez-Freire et al., 1999).

The peptides' safety in terms of their application in foods was also assessed. Both peptides were haemolytic at the highest concentration tested while at the higher end of the MIC range (50 µg/mL) and double the MIC (100 µg/mL), less than 10% haemolysis was observed, a positive attribute if the peptides were to be consumed. Both peptides were sensitive to proteolytic action, an important characteristic for many preservatives. The proteolysis of the peptides supports their gradual degradation after consumption, ensuring that the peptides do not survive the digestion process in the gut.

The evaluation of the peptides' cytotoxicity towards Caco-2 cells found an increase in the cell viability in proportion to the peptide concentration. This behaviour in the presence of the peptides could be linked to their ability to cause cell proliferation. This characteristic has been observed in previous studies in which AMPs were capable of stimulating cell proliferation in dendritic cells, oral epithelial cells and keratinocytes (Ackermann, 2016; Liu et al., 2018; Mi et al., 2018).

Finally, applying the peptides in different beverages matrices revealed that apple juice, cranberry juice and Fanta Orange were suitable food media for their applications. In orange juice, the dense consistency of the liquid could have hindered the peptides' activity against the yeast. In a more viscous matrix like salad dressing, the peptides were able to hinder yeast growth, demonstrating the peptides' potential application in more different food matrices. In order to visualise the effects of the peptides in the preservation of foods with a long shelf life, the peptides incubated for 6 days with the yeast helped illustrate their inhibitory effect long term, as the peptide were capable of maintaining their antiyeast activity over the 6 days.

Conclusion

This study helps illuminate the potential use of these synthetic plant peptides for use in food preservation, as illustrated by their applicable function and potentially safe application in different foods. Although the cost of synthesis can be a disadvantage that still needs to be overcome, the cost and time for the extraction and purification of the natural peptide must also be considered. Thus, the chemical synthesis of known antimicrobial peptides derived from natural sources, as outlined in this study, may represent a novel approach to combatting food spoilage. Such an approach, while currently being too expensive for wide-scale adaption, demonstrates a proof of principle which may become more feasible in the future as the cost of process, such as chemical peptide synthesis, reduce.

Acknowledgements

This work was supported by the Irish Department of Agriculture, Food Institutional Research Measure (F.I.R.M.), Project Reference 15/F/731. A special thank you to Dr Yvonne O'Callaghan for all her help with the cell cultures, Ms. Lilit Ispiryan for her help with the confocal laser scanning microscope and Mr. Jonas Joachim Atzler for the scanning electron microscopy images.

References

Ackermann, M. R. (2016). Inflammation and Healing. In *Pathologic Basis of Veterinary Disease Expert Consult* (pp. 73-131.e2). Elsevier Inc. https://doi.org/10.1016/B978-0-323-35775-3.00003-5

Adem Bahar, A., & Ren, D. (2013). Antimicrobial Peptides. *Pharmaceuticals*, *6*, 1543–1575. https://doi.org/10.3390/ph6121543

Aerts, A. M., François, I. E. J. A., Meert, E. M. K., Li, Q. T., Cammue, B. P. A., & Thevissen, K. (2007). The antifungal activity of RsAFP2, a plant defensin from Raphanus sativus, involves the induction of reactive oxygen species in Candida albicans. *Journal of Molecular Microbiology and Biotechnology*, *13*(4), 243–247. https://doi.org/10.1159/000104753

Ahmad, V., Khan, M. S., Jamal, Q. M. S., Alzohairy, M. A., Al Karaawi, M. A., & Siddiqui, M. U. (2017, January 1). Antimicrobial potential of bacteriocins: in therapy, agriculture and food preservation. *International Journal of Antimicrobial Agents*. Elsevier. https://doi.org/10.1016/j.ijantimicag.2016.08.016

Baldauf, C., Pagel, K., Warnke, S., Von Helden, G., Koksch, B., Blum, V., & Scheffler, M. (2013). How cations change peptide structure. *Chemistry - A European Journal*, *19*(34), 11224–11234. https://doi.org/10.1002/chem.201204554

Betts, G. D., Linton, P., & Betteridge, R. J. (1999). Food spoilage yeasts: Effects of pH, NaCl and temperature on growth. *Food Control*, *10*(1), 27–33. https://doi.org/10.1016/S0956-7135(98)00151-0

Blackburn, C. de W. (2006). *Food spoilage microorganisms*. CRC Press. Retrieved from https://books.google.ie/books?hl=en&lr=&id=trtQAwAAQBAJ&oi=fnd&pg=PP1&dq=

microorganisms+in+food+spoilage+&ots=97LkpxyK-Q&sig=UGVo7ZWFDsQ0OJztc10YT2rPhoY&redir_esc=y#v=onepage&q=microorganisms in food spoilage&f=false

Brown, K. L., & Hancock, R. E. (2006). Cationic host defense (antimicrobial) peptides. *Current Opinion in Immunology*, *18*(1), 24–30. https://doi.org/10.1016/J.COI.2005.11.004

Carvalho, A. O., Machado, O. L. T., Da Cunha, M., Santos, I. S., & Gomes, V. M. (2001). Antimicrobial peptides and immunolocalization of a LTP in Vigna unguiculata seeds. *Plant Physiology and Biochemistry*, *39*(2), 137–146. https://doi.org/10.1016/S0981-9428(00)01230-4

Cleveland, J., Montville, T. J., Nes, I. F., & Chikindas, M. L. (2001, December 4). Bacteriocins: Safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*. Elsevier. https://doi.org/10.1016/S0168-1605(01)00560-8

da Silva Malheiros, P., Daroit, D. J., & Brandelli, A. (2010, June 1). Food applications of liposome-encapsulated antimicrobial peptides. *Trends in Food Science and Technology*. Elsevier. https://doi.org/10.1016/j.tifs.2010.03.003

Dathe, M., & Wieprecht, T. (1999, December 15). Structural features of helical antimicrobial peptides: Their potential to modulate activity on model membranes and biological cells. *Biochimica et Biophysica Acta - Biomembranes*. Elsevier. https://doi.org/10.1016/S0005-2736(99)00201-1

De Samblanx, G. W., Goderis, I. J., Thevissen, K., Raemaekers, R., Fant, F., Borremans, F., … Broekaert, W. F. (1997a). Mutational analysis of a plant defensin from radish (Raphanus sativus L.) reveals two adjacent sites important for antifungal activity. *Journal of Biological Chemistry*, *272*(2), 1171–1179. https://doi.org/10.1074/jbc.272.2.1171

De Samblanx, G. W., Goderis, I. J., Thevissen, K., Raemaekers, R., Fant, F., Borremans, F., … Broekaert, W. F. (1997b). Mutational analysis of a plant defensin from radish (Raphanus sativus L.) reveals two adjacent sites important for antifungal activity. *Journal of Biological Chemistry*, *272*(2), 1171–1179. https://doi.org/10.1074/jbc.272.2.1171

De Vuyst, L., & Leroy, F. (2007). Bacteriocins from lactic acid bacteria: Production, purification, and food applications. In *Journal of Molecular Microbiology and Biotechnology* (Vol. 13, pp. 194–199). Karger Publishers. https://doi.org/10.1159/000104752

de W. Blackburn, C. (2010). *Food spoilage microorganisms*. *Food spoilage microorganisms*. https://doi.org/10.1533/9781845691417

Djiadeu, P., Azzouz, D., Khan, M. A., Kotra, L. P., Sweezey, N., & Palaniyar, N. (2017). Ultraviolet irradiation increases green fluorescence of dihydrorhodamine (DHR) 123: false-positive results for reactive oxygen species generation. *Pharmacology Research and Perspectives*, *5*(2), 1–11. https://doi.org/10.1002/prp2.303

Enríquez-Freire, E., López, R., & Peña, A. (1999). Potassium ion efflux induced by cationic compounds in yeast. *Biochimica et Biophysica Acta - Biomembranes*, *1418*(1), 147–157. https://doi.org/10.1016/S0005-2736(99)00015-2

Fant, F., Vranken, W., Broekaert, W., & Borremans, F. (1998). Determination of the three-dimensional solution structure of Raphanus sativus antifungal protein 1 by 1H NMR. *Journal of Molecular Biology*, *279*(1), 257–270. https://doi.org/10.1006/jmbi.1998.1767

Fleet, G. H., & Mian, M. A. (1987). The occurrence and growth of yeasts in dairy products. *International Journal of Food Microbiology*, *4*(2), 145–155. https://doi.org/10.1016/0168-1605(87)90021-3

Fry, D. E. (2018). Antimicrobial peptides. *Surgical Infections*, *19*(8), 804–811. https://doi.org/10.1089/sur.2018.194

Fung, D. Y. C. (2009). Food Spoilage, Preservation and Quality Control. In *Encyclopedia of Microbiology* (pp. 54–79). Elsevier. https://doi.org/10.1016/b978-012373944-5.00122-x

Garvey, M., Meehan, S., Gras, S. L., Schirra, H. J., Craik, D. J., Van Der Weerden, N. L., … Carver, J. A. (2013). A radish seed antifungal peptide with a high amyloid fibril-forming propensity. *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1834*(8), 1615–1623. https://doi.org/10.1016/j.bbapap.2013.04.030

Goyal, R. K., & Mattoo, A. K. (2016). Plant antimicrobial peptides. In *Host Defense Peptides and Their Potential as Therapeutic Agents* (Vol. 59, pp. 111–136). Springer. https://doi.org/10.1007/978-3-319-32949-9_5

Haag, A. F., Kerscher, B., Dall'Angelo, S., Sani, M., Longhi, R., Baloban, M., … Ferguson, G. P. (2012). Role of cysteine residues and disulfide bonds in the activity of a legume root nodule-specific, cysteine-rich peptide. *Journal of Biological Chemistry*, *287*(14), 10791–10798. https://doi.org/10.1074/jbc.M111.311316

Hancock, R. E. ., & Diamond, G. (2000). The role of cationic antimicrobial peptides in innate host defences. *Trends in Microbiology*, *8*(9), 402–410. https://doi.org/10.1016/S0966-842X(00)01823-0

Hancock, R. E. W. (1999). Host Defence (Cationic) Peptides. *Drugs*, *57*(4), 469–473. https://doi.org/10.2165/00003495-199957040-00002

Hayes, B. M. E., Bleackley, M. R., Wiltshire, J. L., Anderson, M. A., Traven, A., & Van Der Weerden, N. L. (2013). Identification and mechanism of action of the plant defensin nad1 as a new member of the antifungal drug arsenal against candida albicans. *Antimicrobial*

Agents and Chemotherapy, *57*(8), 3667–3675. https://doi.org/10.1128/AAC.00365-13

Hong, S. Y., Park, T. G., & Lee, K. H. (2001). The effect of charge increase on the specificity and activity of a short antimicrobial peptide. *Peptides*, *22*(10), 1669–1674. https://doi.org/10.1016/S0196-9781(01)00502-2

Houtsma, P. C., de Wit, J. C., & Rombouts, F. M. (1993, December). Minimum inhibitory concentration (MIC) of sodium lactate for pathogens and spoilage organisms occurring in meat products. *International Journal of Food Microbiology*. https://doi.org/10.1016/0168-1605(93)90169-H

Jang, W. S., Kim, H. K., Lee, K. Y., Kim, S. A., Han, Y. S., & Lee, I. H. (2006). Antifungal activity of synthetic peptide derived from halocidin, antimicrobial peptide from the tunicate, Halocynthia aurantium. *FEBS Letters*, *580*(5), 1490–1496. https://doi.org/10.1016/j.febslet.2006.01.041

Jenssen, H., Hamill, P., & Hancock, R. E. W. (2006). Peptide antimicrobial agents. *Clinical Microbiology Reviews*, *19*(3), 491–511. https://doi.org/10.1128/CMR.00056-05

Jiang, Z., Vasil, A. I., Hale, J. D., Hancock, R. E. W., Vasil, M. L., & Hodges, R. S. (2008). Effects of net charge and the number of positively charged residues on the biological activity of amphipathic alpha-helical cationic antimicrobial peptides. *Biopolymers*, *90*(3), 369–383. https://doi.org/10.1002/bip.20911

Kalathenos, P., Sutherland, J. P., & Roberts, T. A. (1995). Resistance of some wine spoilage yeasts to combinations of ethanol and acids present in wine. *Journal of Applied Bacteriology*, *78*(3), 245–250. https://doi.org/10.1111/j.1365-2672.1995.tb05023.x

Koczulla, A. R., & Bals, R. (2003). Antimicrobial peptides: Current status and therapeutic potential. *Drugs*. https://doi.org/10.2165/00003495-200363040-00005

Korkeala, H. J., & Johanna Björkroth, K. (1997). Microbiological spoilage and contamination of vacuum-packaged cooked sausages. *Journal of Food Protection*. https://doi.org/10.4315/0362-028X-60.6.724

Kuanyshev, N., Adamo, G. M., Porro, D., & Branduardi, P. (2017). The spoilage yeast Zygosaccharomyces bailii: Foe or friend? *Yeast*, *34*(9), 359–370. https://doi.org/10.1002/yea.3238

Kumar, P., Kizhakkedathu, J. N., & Straus, S. K. (2018, January 19). Antimicrobial peptides: Diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. *Biomolecules*. Multidisciplinary Digital Publishing Institute. https://doi.org/10.3390/biom8010004

Lay, F., & Anderson, M. (2005). Defensins - Components of the Innate Immune System in Plants. *Current Protein & Peptide Science*, *6*(1), 85–101. https://doi.org/10.2174/1389203053027575

Lee, W., & Lee, D. G. (2015). Fungicidal mechanisms of the antimicrobial peptide Bac8c. *Biochimica et Biophysica Acta - Biomembranes*, *1848*(2), 673–679. https://doi.org/10.1016/j.bbamem.2014.11.024

Liu, N., Guan, S., Wang, H., Li, C., Cheng, J., Yu, H., … Pan, Y. (2018). The Antimicrobial Peptide Nal-P-113 Exerts a Reparative Effect by Promoting Cell Proliferation, Migration, and Cell Cycle Progression. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/7349351

Mahlapuu, M., Håkansson, J., Ringstad, L., & Björn, C. (2016). Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. *Frontiers in Cellular and Infection Microbiology*, *6*(December), 1–12. https://doi.org/10.3389/fcimb.2016.00194

Maróti, G., Downie, J. A., & Kondorosi, É. (2015, August 1). Plant cysteine-rich peptides that inhibit pathogen growth and control rhizobial differentiation in legume nodules. *Current Opinion in Plant Biology*. Elsevier Current Trends. https://doi.org/10.1016/j.pbi.2015.05.031

Mayoral, M. B., Martín, R., Sanz, A., Hernández, P. E., González, I., & García, T. (2005). Detection of Kluyveromyces marxianus and other spoilage yeasts in yoghurt using a PCR-culture technique. *International Journal of Food Microbiology*, *105*(1), 27–34. https://doi.org/10.1016/j.ijfoodmicro.2005.06.006

Mi, B., Liu, J., Liu, Y., Hu, L., Liu, Y., Panayi, A. C., … Liu, G. (2018). The Designer Antimicrobial Peptide A-hBD-2 Facilitates Skin Wound Healing by Stimulating Keratinocyte Migration and Proliferation. *Cellular Physiology and Biochemistry*, *51*(2), 647–663. https://doi.org/10.1159/000495320

Murtey, M. Das, & Ramasamy, P. (2016). Sample Preparations for Scanning Electron Microscopy – Life Sciences. In *Modern Electron Microscopy in Physical and Life Sciences*. InTech. https://doi.org/10.5772/61720

Neuhaus, J. M. (1999). Plant chitinases (PR-3, PR-4, PR-8, PR-11). In *Pathogenesis-Related Proteins in Plants* (Vol. 216, pp. 77–105). Springer-Verlag. https://doi.org/10.1201/9781420049299

Okamoto, M., Mitsuhara, I., Ohshima, M., Natori, S., & Ohashi, Y. (1998). Enhanced expression of an antimicrobial peptide sarcotoxin IA by GUS fusion in transgenic tobacco plants. *Plant and Cell Physiology*, *39*(1), 57–63. https://doi.org/10.1093/oxfordjournals.pcp.a029289

Osborn, R. W., De Samblanx, G. W., Thevissen, K., Goderis, I., Torrekens, S., Van Leuven, F., … Broekaert, W. F. (1995). Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae. *FEBS Letters*, *368*(2), 257–262. https://doi.org/10.1016/0014-5793(95)00666-W

Pelegrini, P. B., & Franco, O. L. (2005, November 1). Plant γ-thionins: Novel insights on the mechanism of action of a multi-functional class of defense proteins. *International Journal of Biochemistry and Cell Biology*. Pergamon. https://doi.org/10.1016/j.biocel.2005.06.011

Peña, A., Sánchez, N. S., & Calahorra, M. (2013). Effects of chitosan on candida albicans: Conditions for its antifungal activity. *BioMed Research International*, *2013*, 1–15. https://doi.org/10.1155/2013/527549

Sato, H., & Feix, J. B. (2008). Lysine-enriched cecropin-mellitin antimicrobial peptides with enhanced selectivity. *Antimicrobial Agents and Chemotherapy*, *52*(12), 4463–4465. https://doi.org/10.1128/AAC.00810-08

Schmidt, M., Arendt, E. K., & Thery, T. L. C. (2019). Isolation and characterisation of the antifungal activity of the cowpea defensin Cp-thionin II. *Food Microbiology*, *82*, 504–514. https://doi.org/10.1016/j.fm.2019.03.021

SD, S. (2014). NET CHARGE, HYDROPHOBICITY AND SPECIFIC AMINO ACIDS CONTRIBUTE TO THE ACTIVITY OF ANTIMICROBIAL PEPTIDES. *Journal of Health and Translational Medicine*, *17*(1), 1–7. https://doi.org/10.22452/jummec.vol17no1.1

Seo, M. D., Won, H. S., Kim, J. H., Mishig-Ochir, T., & Lee, B. J. (2012, October 18). Antimicrobial peptides for therapeutic applications: A review. *Molecules*. Molecular Diversity Preservation International. https://doi.org/10.3390/molecules171012276

Shwaiki, L. N., Arendt, E. K., Lynch, K. M., & Thery, T. L. C. (2019). Inhibitory effect of four novel synthetic peptides on food spoilage yeasts. *International Journal of Food Microbiology*, *300*, 43–52. https://doi.org/10.1016/J.IJFOODMICRO.2019.04.005

Tailor, R. H., Acland, D. P., Attenborough, S., Cammue, B. P. A., Evans, I. J., Osborn, R. W., … Broekaert, W. F. (1997). A novel family of small cysteine-rich antimicrobial peptides from seed of Impatiens balsamina is derived from a single precursor protein. *Journal of Biological Chemistry*, *272*(39), 24480–24487. https://doi.org/10.1074/jbc.272.39.24480

Tang, S. S., Prodhan, Z. H., Biswas, S. K., Le, C. F., & Sekaran, S. D. (2018, October 1). Antimicrobial peptides from different plant sources: Isolation, characterisation, and purification. *Phytochemistry*. Pergamon. https://doi.org/10.1016/j.phytochem.2018.07.002

Terras, F. R. G., Schoofs, H. M. E., De Bolle, M. F. C., Van Leuven, F., Rees, S. B., Vanderleyden, J., … Broekaert, W. F. (1992). Analysis of two novel classes of plant antifungal proteins from radish (Raphanus sativus L.) seeds. *Journal of Biological Chemistry*, *267*(22), 15301–15309. https://doi.org/10.1104/PP.108.4.1353

Thery, T., & Arendt, E. K. (2018). Antifungal activity of synthetic cowpea defensin Cp-thionin II and its application in dough. *Food Microbiology*, *73*, 111–121. https://doi.org/10.1016/j.fm.2018.01.006

Thery, T., Shwaiki, L. N., O'Callaghan, Y. C., O'Brien, N. M., & Arendt, E. K. (2019). Antifungal activity of a de novo synthetic peptide and derivatives against fungal food contaminants. *Journal of Peptide Science*, *25*(1), e3137. https://doi.org/10.1002/psc.3137

Thomas, D. S., & Davenport, R. R. (1985). Zygosaccharomyces bailii - a profile of characteristics and spoilage activities. *Food Microbiology*, *2*(2), 157–169. https://doi.org/10.1016/S0740-0020(85)80008-3

Titarenko, E., López-Solanilla, E., García-Olmedo, F., & Rodríguez-Palenzuela, P. (1997). Mutants of Ralstonia (Pseudomonas) solanacearum sensitive to antimicrobial peptides are altered in their lipopolysaccharide structure and are avirulent in tobacco. *Journal of Bacteriology*, *179*(21), 6699–6704. https://doi.org/10.1128/jb.179.21.6699-6704.1997

Van Der Weerden, N. L., Bleackley, M. R., & Anderson, M. A. (2013, October 5). Properties and mechanisms of action of naturally occurring antifungal peptides. *Cellular and Molecular Life Sciences*. Springer Basel. https://doi.org/10.1007/s00018-013-1260-1

Walkenhorst, W. F., Klein, J. W., Vo, P., & Wimley, W. C. (2013). PH dependence of microbe sterilization by cationic antimicrobial peptides. *Antimicrobial Agents and Chemotherapy*, *57*(7), 3312–3320. https://doi.org/10.1128/AAC.00063-13

Wang, K., Dang, W., Xie, J., Zhu, R., Sun, M., Jia, F., … Wang, R. (2015). Antimicrobial peptide protonectin disturbs the membrane integrity and induces ROS production in yeast cells. *Biochimica et Biophysica Acta - Biomembranes*, *1848*(10), 2365–2373. https://doi.org/10.1016/j.bbamem.2015.07.008

Westall, S., & Filtenborg, O. (1998). Spoilage yeasts of decorated soft cheese packed in modified atmosphere. *Food Microbiology*, *15*(2), 243–249. https://doi.org/10.1006/fmic.1997.0162

Whitlow, M., & Teeter, M. M. (1985). Energy minimization for tertiary structure prediction of homologous proteins: Ai-purothionin and viscotoxin a3 models from crambin. *Journal of Biomolecular Structure and Dynamics*, *2*(4), 831–848. https://doi.org/10.1080/07391102.1985.10506327

Wu, G., Ding, J., Li, H., Li, L., Zhao, R., Shen, Z., … Xi, T. (2008). Effects of cations and PH on antimicrobial activity of thanatin and s-thanatin against Escherichia coli ATCC25922 and B. subtilis ATCC 21332. *Current Microbiology*, *57*(6), 552–557.

https://doi.org/10.1007/s00284-008-9241-6

Yeaman, M. R., & Yount, N. Y. (2003, March 1). Mechanisms of antimicrobial peptide action and resistance. *Pharmacological Reviews*. American Society for Pharmacology and Experimental Therapeutics. https://doi.org/10.1124/pr.55.1.2

Zaiou, M. (2007, March 20). Multifunctional antimicrobial peptides: Therapeutic targets in several human diseases. *Journal of Molecular Medicine*. Springer-Verlag. https://doi.org/10.1007/s00109-006-0143-4

Zhang, Y., & Lewis, K. (1997). Fabatins: New antimicrobial plant peptides. *FEMS Microbiology Letters*, *149*(1), 59–64. https://doi.org/10.1016/S0378-1097(97)00054-2

Tables

Table 1: Sequences of Rs-AFP1 and Rs-AFP2

Peptide	Amino Acid Sequence
Rs-AFP1	QKLC**E**RPSGTWSGVCGNNNACKNQCI**N**LEKARHGSCNYVFPAHKCICYFPC
Rs-AFP2	QKLC**Q**RPSGTWSGVCGNNNACKNQCI**R**LEKARHGSCNYVFPAHKCICYFPC

Table 2: Ranges of minimum inhibitory concentrations of Rs-AFP1 and Rs-AFP2 against Zygosaccharomyces bailli Sa1403, Zygosaccharomyces rouxii ATCC14679, Saccharomyces cerevisiae Baker's yeast, Kluyveromyces lactis ATCC56498 and Debaromyces hansenii CBS2334.

	Zygosaccharomyces bailli Sa1403	***Zygosaccharomyces rouxii ATCC14679***	***Saccharomyces cerevisiae Baker's yeast***	***Kluyveromyces lactis ATCC56498***	***Debaromyces hansenii CBS2334***
Rs-AFP1	MIC range of 25 to 50 µg/mL – Fungistatic	No inhibition	No inhibition	No inhibition	No inhibition
Rs-AFP2	MIC range of 25 to 50 µg/mL - Fungicidal	MIC range of 50 to 100 µg/mL - Fungistatic	No inhibition	No inhibition	MIC range of 50 to 100 µg/mL - Fungistatic

Table 3: The effect of the medium pH change on Rs-AFP1 and Rs-AFP2 and their antiyeast activity.

* No yeast growth was observed due to the yeast's inability to grow at such high pH.

	pH 3	pH 5	pH 7	pH 9	pH 11
Rs-AFP1	No inhibition	Full inhibition due to peptide activity	No inhibition	No yeast growth	No yeast growth*
Rs-AFP2	No inhibition	Full inhibition due to peptide activity	No inhibition	No yeast growth	No yeast growth*

Table 4: Antiyeast effect of Rs-AFP1 and Rs-AFP2 on Z. bailii in different beverages.

	Cranberry Juice	Fanta Orange	Apple Juice	Orange Juice
Rs-AFP1	Full inhibition†	Full inhibition†	Inhibition at 200 and 400 µg/mL	No inhibition
Rs-AFP2	Full inhibition†	Full inhibition†	Inhibition at 200 and 400 µg/mL	No inhibition

†Full inhibition at all concentrations tested.

Figure Captions

Figure 1. Colony count assay demonstrating the rate of *Z. bailii* inhibition caused by Rs-AFP1 (A) and Rs-AFP2 (B). Yeast growth reduced after only 1 hr of incubation in the presence of both peptides at the highest concentrations, in comparison to the control, which showed a steady increase in growth over the 6 hrs.

Figure 2. Stability of Rs-AFP2 (A) and Rs-AFP1 (B) in $MgCl_2$ and KCl at different concentrations. The lowest concentration of $MgCl_2$ resulted in no negative effects of Rs-AFP2's antiyeast activity as seen from inhibition of *Z. bailii* at 100 µg/mL.

Figure 3. SEM images of *Z. bailii* in the absence of peptide (A), and with Rs-AFP1 after 0 hrs (B) and 4 hrs (C) incubation. Rs-AFP2 after 0 hrs (D) and 4 hrs (E) showed similar images.

Figure 4. Percentage of haemolysis by Rs-AFP1 and Rs-AFP2 on sheep erythrocytes.

Figure 5. Increase in the viability of Caco-2 cells in the presence of increasing concentrations of Rs-AFP1 (A) and Rs-AFP2 (B) (0-600 µg/mL).

Figure 6. The effect of Rs-AFP1 and Rs-AFP2 on *Z. bailii* growth in a sample of salad dressing after the immediate incubation of the peptides with the yeast.

Figure 1

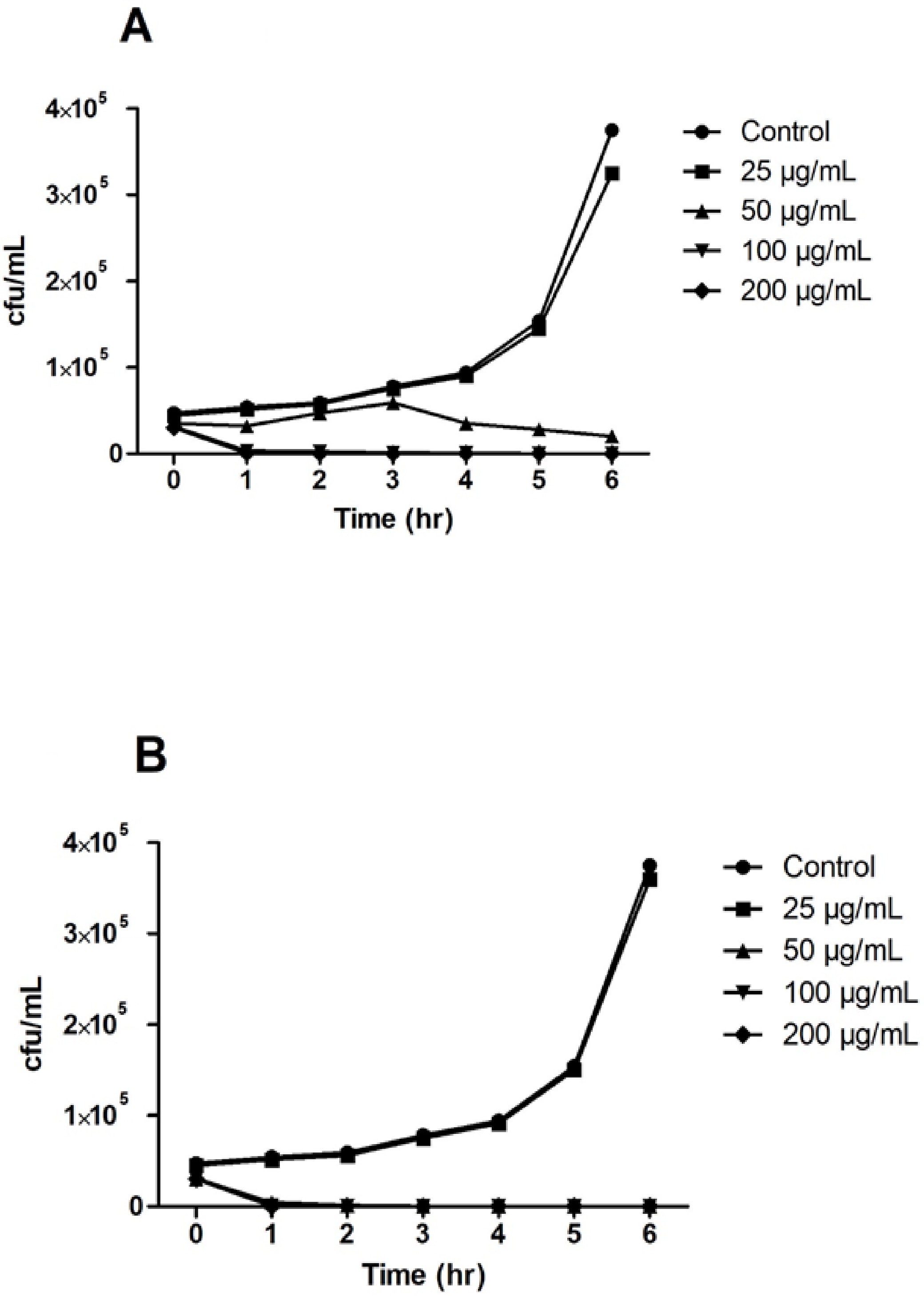

A
cfu/mL
4×10^5
3×10^5
2×10^5
1×10^5
0
0 1 2 3 4 5 6
Time (hr)
Control
25 µg/mL
50 µg/mL
100 µg/mL
200 µg/mL
B
cfu/mL
4×10^5
3×10^5
2×10^5
1×10^5
0
0 1 2 3 4 5 6
Time (hr)
Control
25 µg/mL
50 µg/mL
100 µg/mL
200 µg/mL

Figure 2

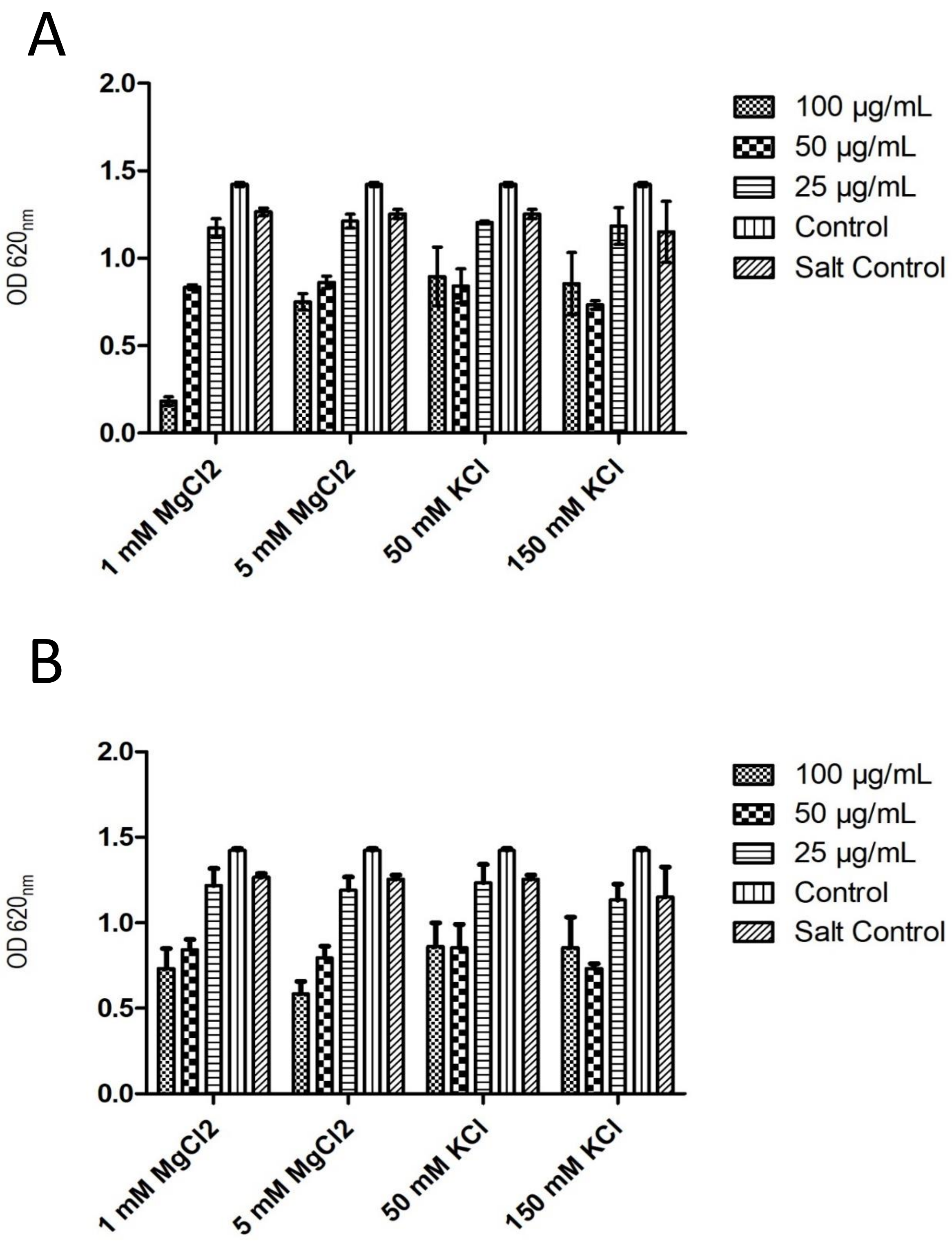

A
2.0
1.5
1.0
0.5
0.0
OD 620nm
1 mM MgCl2
5 mM MgCl2
50 mM KCl
150 mM KCl
100 µg/mL
50 µg/mL
25 µg/mL
Control
Salt Control
B
2.0
1.5
1.0
0.5
0.0
OD 620nm
1 mM MgCl2
5 mM MgCl2
50 mM KCl
150 mM KCl
100 µg/mL
50 µg/mL
25 µg/mL
Control
Salt Control

Figure 3

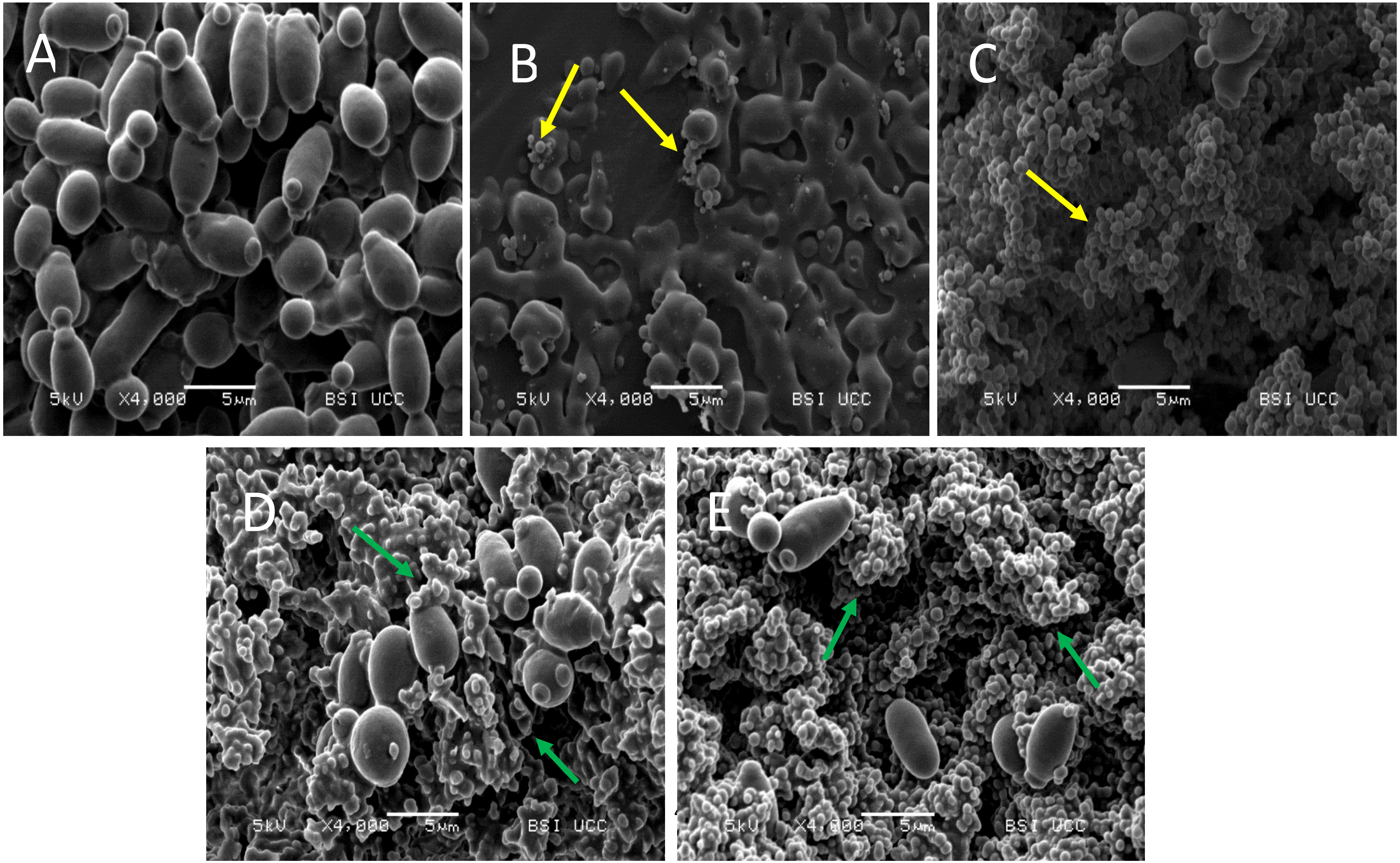

A
5kV X4,000 5µm BSI UCC
B
5kV X4,000 5µm BSI UCC
C
5kV X4,000 5µm BSI UCC
D
5kV X4,000 5µm BSI UCC
E
5kV X4,000 5µm BSI UCC

Figure 4

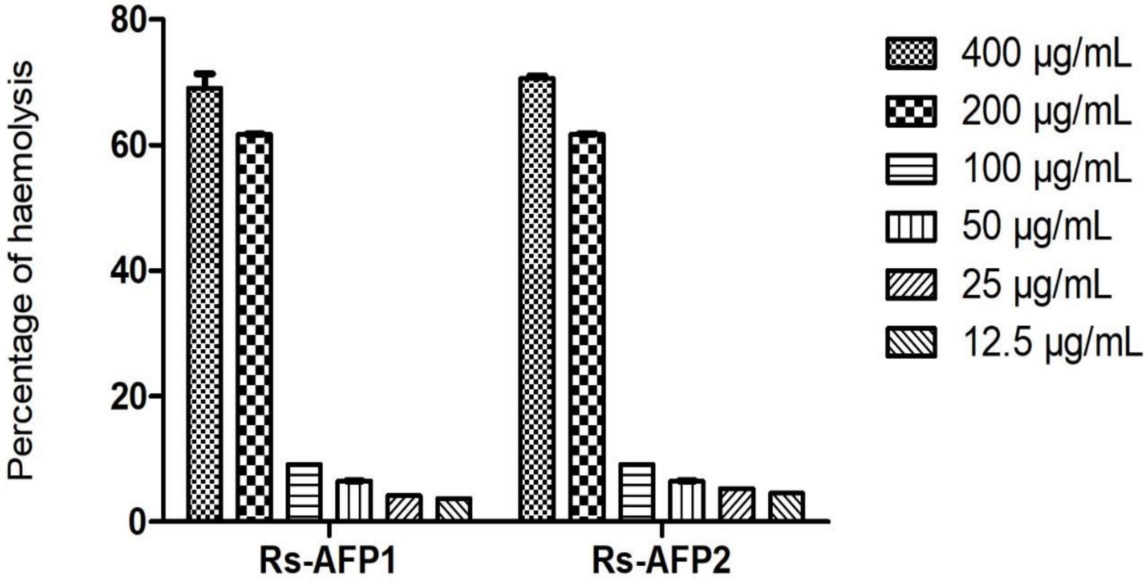
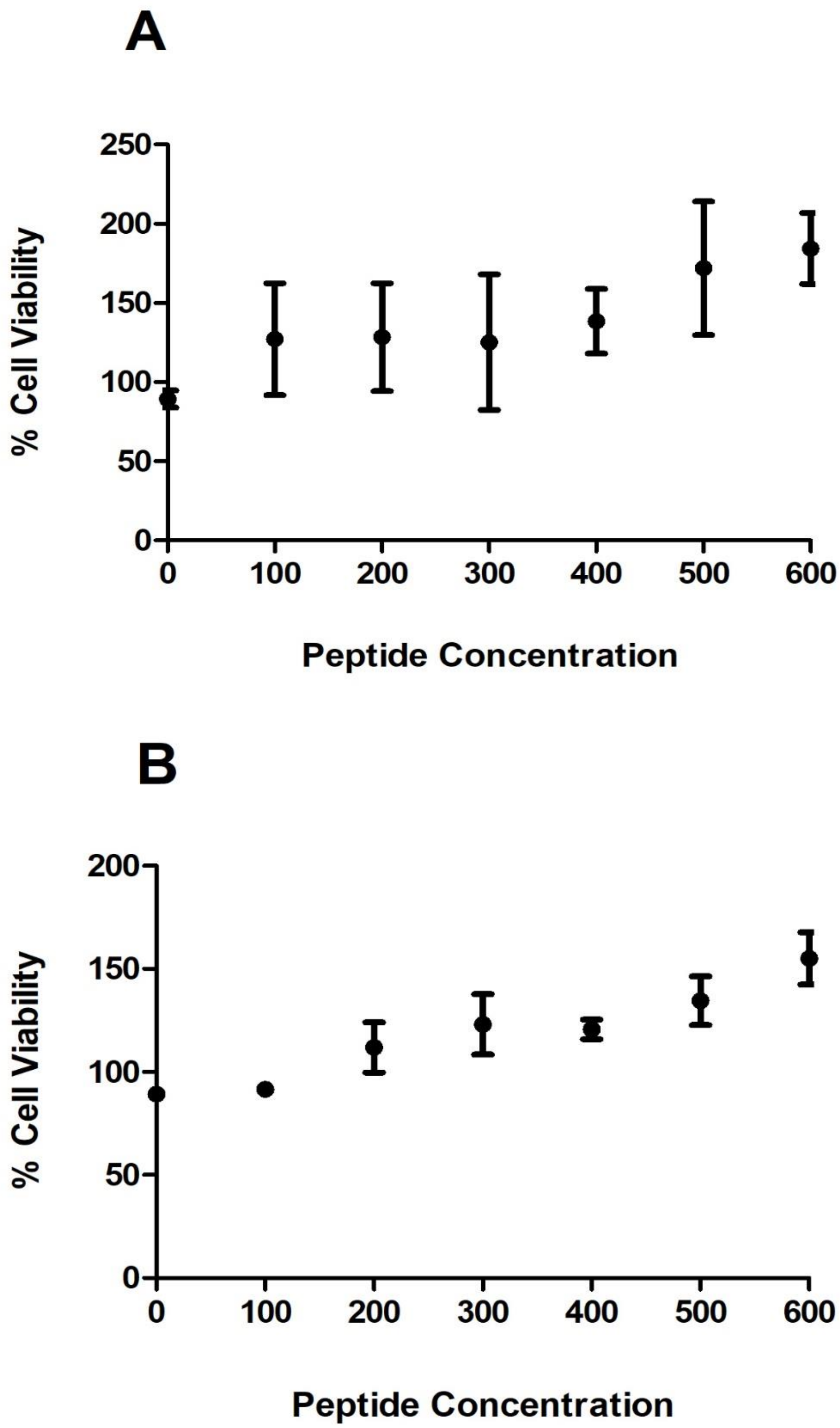
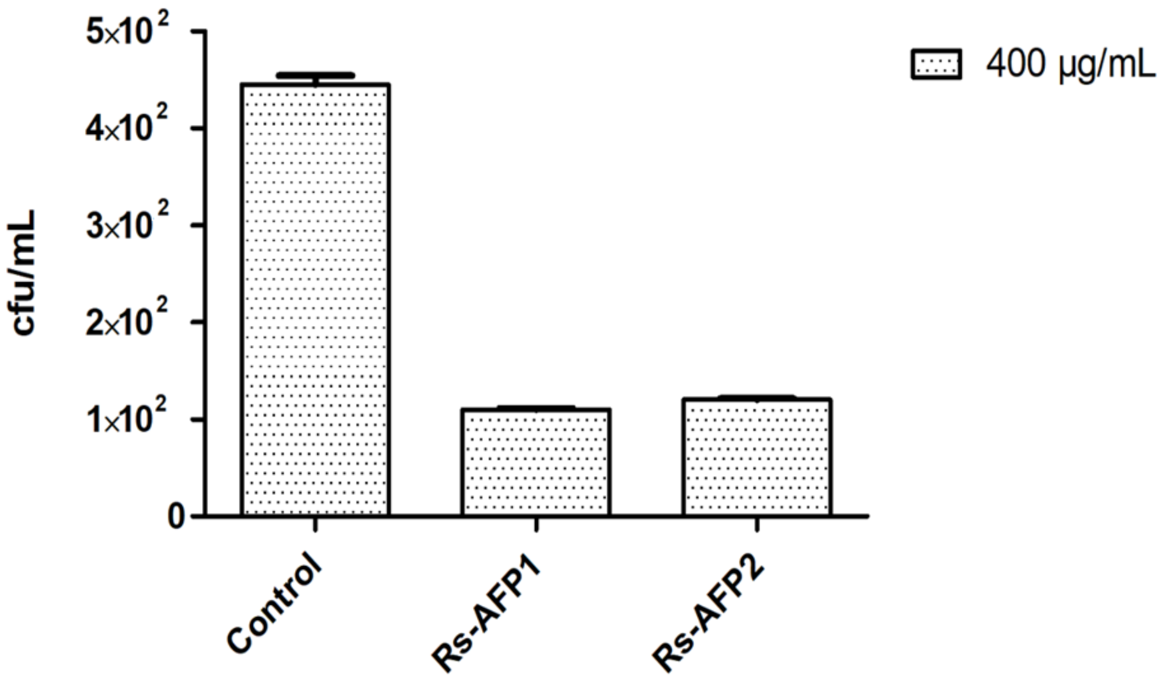

Percentage of haemolysis
80
60
40
20
0
Rs-AFP1
Rs-AFP2
400 µg/mL
200 µg/mL
100 µg/mL
50 µg/mL
25 µg/mL
12.5 µg/mL

Figure 5

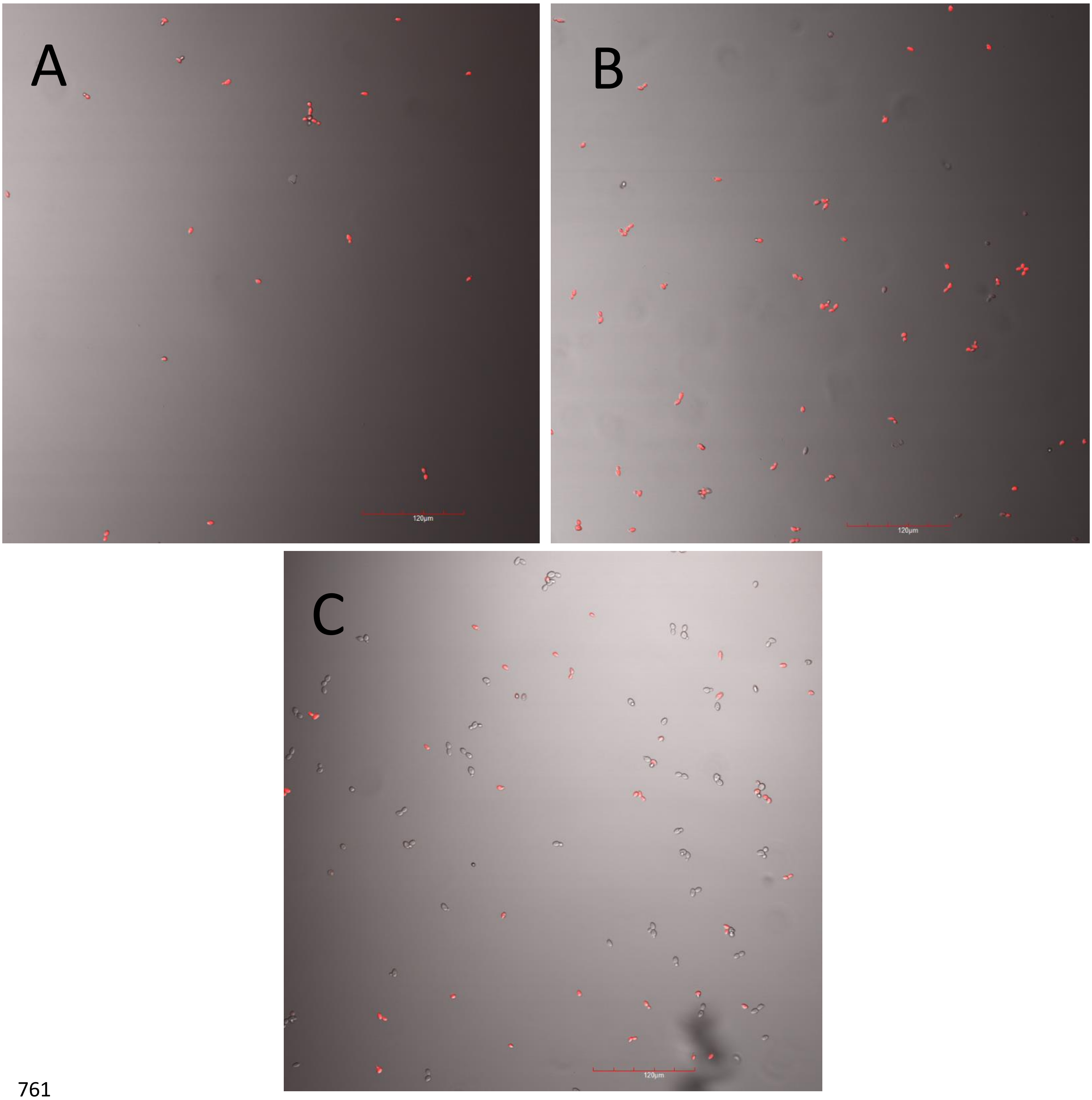

A
% Cell Viability
250
200
150
100
50
0
0
100
200
300
400
500
600
Peptide Concentration
B
% Cell Viability
200
150
100
50
0
0
100
200
300
400
500
600
Peptide Concentration

758 **Figure 6**



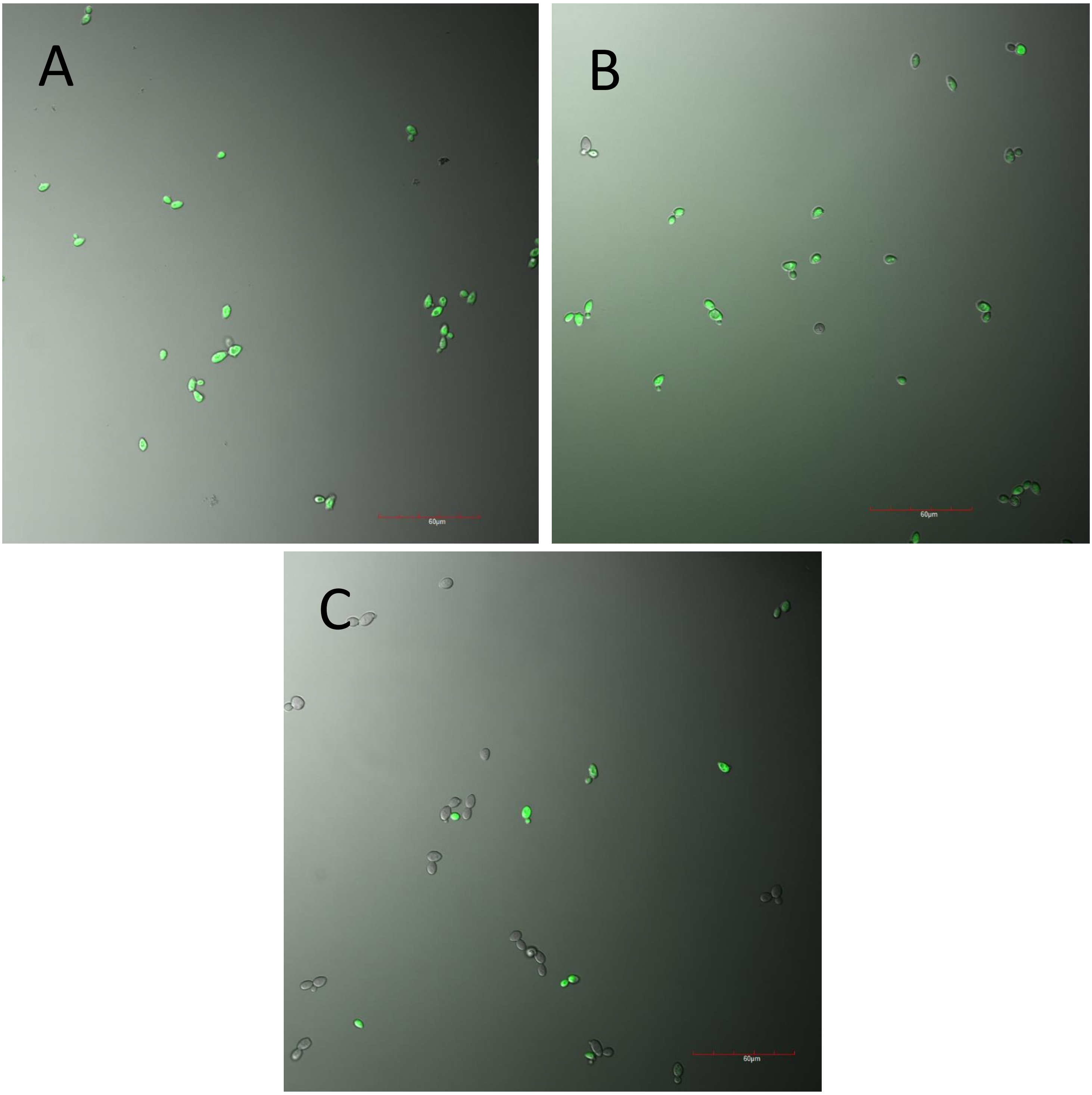
759

Supplementary Figure 1



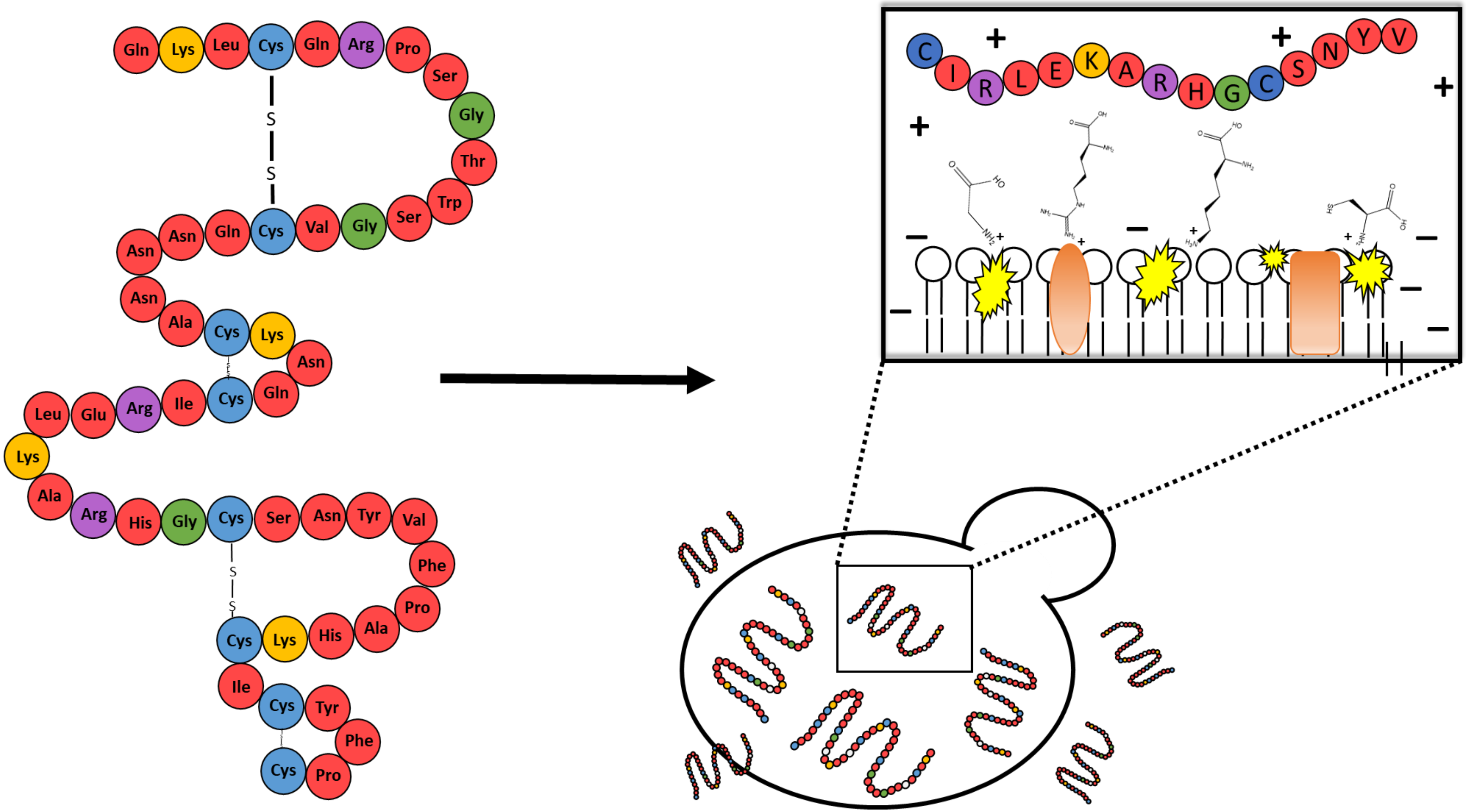
Supplementary figure 1. CLSM images showing the impact of Rs-AFP2 on *Z. bailii* cell membrane at 400 µg/mL (A), 200 µg/mL (B) and 100 µg/mL (C). The highest peptide concentration resulted in complete uptake of the dye caused by the permeabilisation of the yeast membrane, suggesting cell death. The level of permeabilisation is seen to reduce with decreasing peptide concentration.

Supplementary figure 2



Supplementary figure 2. CLSM images showing the overproduction of ROS in *Z. bailii* as a result of Rs-AFP2 at 400 µg/mL (A), 200 µg/mL (B) and 100 µg/mL (C). Fluorescence of the cells indicate oxidation of Dihydrorhodamine 123 dye to rhodamine 123 in the presence of reactive oxygen species.

Supplementary figure 3



Supplementary figure 3. Schematic representation of the mechanism of action of the peptides against yeast. A simple secondary structure of Rs-AFP2 was constructed, revealing 4 disulfide bonds between the 8 cysteine residues present. The 4 main amino acid residues thought to be effectors are highlighted in blue, green, purple and yellow, representing Cys, Gly, Arg and Lys, respectively. The peptide accumulates on the surface of the yeast membrane with interaction of the positively charged amino acid residues with the negatively charged yeast membrane, causing membrane damage.